The Pds1 anaphase inhibitor and Mec1 kinase define distinct checkpoints coupling S phase with mitosis in budding yeast

Duncan J. Clarke*†, Marisa Segal*†, Guillaume Mondésert‡ and Steven I. Reed*

In most eukaryotic cells, DNA replication is confined to S phase of the cell cycle [1]. During this interval, S-phase checkpoint controls restrain mitosis until replication is complete [2]. In budding yeast, the anaphase inhibitor Pds1p has been associated with the checkpoint arrest of mitosis when DNA is damaged or when mitotic spindles have formed aberrantly [3,4], but not when DNA replication is blocked with hydroxyurea (HU). Previous studies have implicated the protein kinase Mec1p in S-phase checkpoint control [5]. Unlike *mec1* mutants, *pds1* mutants efficiently inhibit anaphase when replication is blocked. This does not, however, exclude an essential S-phase checkpoint function of Pds1 beyond the early S-phase arrest point of a HU block. Here, we show that Pds1p is an essential component of a previously unsuspected checkpoint control system that couples the completion of S phase with mitosis. Further, the S-phase checkpoint comprises at least two distinct pathways. A Mec1p-dependent pathway operates early in S phase, but a Pds1p-dependent pathway becomes essential part way through S phase.

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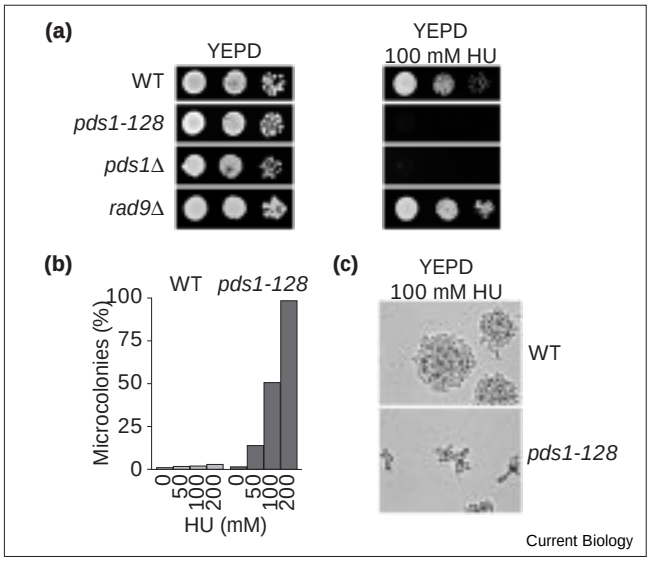
Results and discussion

In budding yeast, initiation of anaphase is controlled by the ubiquitin-dependent degradation of the anaphase inhibitor Pds1p. This process constitutes a target of late cycle checkpoint controls [6]. To address whether Pds1p is required for S-phase checkpoint control, we adopted two approaches. First, we used a hypomorphic *pds1* allele, *pds1-128*, that causes a less severe temperature sensitivity than a null allele and is, therefore, more amenable to the study of cell-cycle events in synchronous populations. Although the restrictive temperature for growth of *pds1-128* cells is 37°C, DNA damage and spindle assembly checkpoint defects are apparent at 26°C, comparable to those previously described for the *pds1-1* mutant [3,4]. Although a replication block induced by 400 mM HU caused *pds1-128* and *pds1Δ* cells to checkpoint-arrest (data not shown), these mutants were highly sensitive to non-replication-arresting doses of HU (50–100 mM; Figure 1a). On solid medium containing 100 mM HU, *pds1-128* mutants formed microcolonies (Figure 1b,c). In liquid medium containing 100 mM HU, at least 50% of *pds1-128* cells lost viability per generation (see Supplementary material published with this article on the internet). Crucially, *rad9Δ* cells, defective for DNA damage checkpoint control, were not sensitive to 100 mM HU (Figure 1a). Hence, the sensitivity of *pds1* mutants does not result from a Rad9p-dependent DNA damage checkpoint defect.

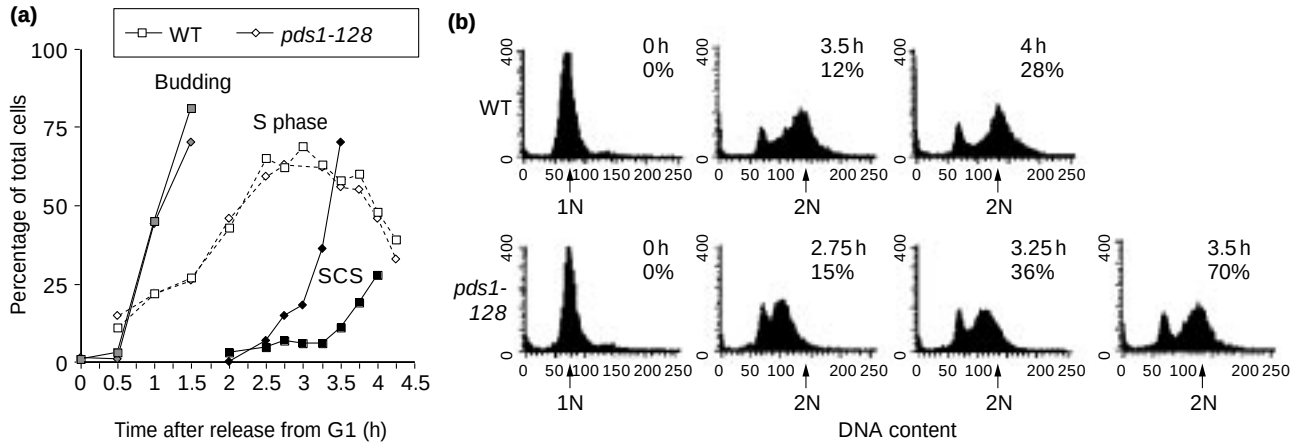
Second, we performed kinetic studies in which the coupling of S phase with mitosis was challenged by partial inhibition of replication. Normally, replication is completed before short G2 spindles form [7]. In the presence of 100 mM HU, replication proceeds at a reduced rate [7]. Only once cells have budded and formed short mitotic spindles must anaphase be delayed to allow the completion of replication. Under these conditions, loss of S-phase

Figure 1



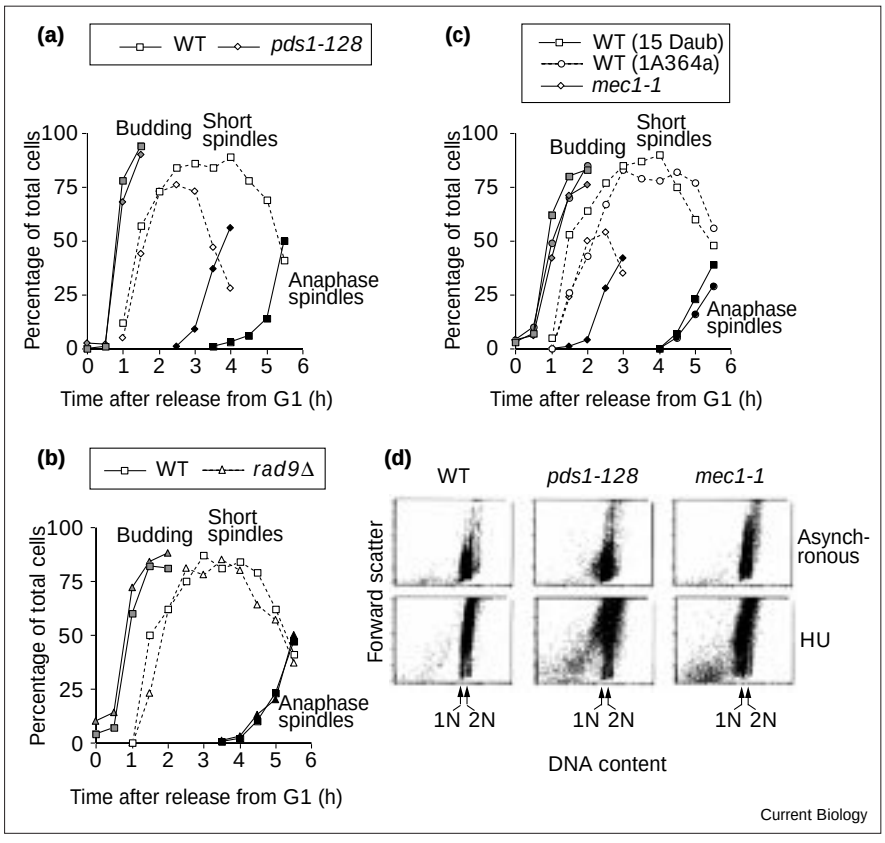
Sensitivity of *pds1* mutants to HU. Serial dilutions of mid-log cells from wild type (WT), *pds1* or *rad9* mutants were spotted onto solid YEPD medium or onto YEPD containing HU and grown at 30°C (25°C for *pds1Δ*). (a) Spot growth was recorded after 2–3 days. After 24 h, microcolonies were (b) counted and (c) photographed.

Figure 2



Loss of sister centromere cohesion during S phase in *pds1-128* cells. Wild-type (WT) and *pds1-128* cells were arrested in G1 with 200 ng/ml α factor (at least 90% unbudded cells), then released into rich medium containing 100 mM HU at 30°C. (a) Cell aliquots were taken at given time intervals for scoring budding index (gray symbols), sister centromere separation (SCS) for chromosome IV (black symbols), the percentage of cells in S phase (open symbols), and for FACScan analysis of DNA content. (b) Histograms show the DNA content of cells in samples at selected time points (the time following α factor release and the percentage of separated centromeres are indicated above the corresponding graph). When *pds1-128* cells divided before DNA replication was complete, nuclear division was unequal. This resulted in G1 cells with greater than 1N DNA content; the next S phase further increased the DNA content of these cells, a fact apparent late in this time course (notice the sub-2N peak for the *pds1-128* cells at 3.5 h).

Figure 3



Distinct checkpoint control defects in *pds1* and *mec1* mutants. Strains were G1-arrested as in Figure 2, then released into rich medium containing 100 mM HU at 26°C. Cell aliquots were taken at given time intervals to score budding index (gray symbols), short spindle formation (open symbols) and spindle elongation (black symbols), and for FACScan analysis of DNA content. Each strain replicated DNA with similar kinetics (the kinetics of replication in *mec1-1* cells could not be determined because these cells began anaphase before much DNA had been replicated; data not shown). (a) Wild-type (WT) and *pds1-128* cells. (b) Wild-type and *rad9Δ* cells. (c) Wild-type (in both the 15Daub and A364a genetic backgrounds) and *mec1-1* (A364a genetic background) cells. (d) Dot plots of DNA content versus forward scatter for wild-type, *pds1-128* and *mec1-1* cells grown in rich media with or without 100 mM HU for three generations following release from α factor. The positions of 1N and 2N DNA content are indicated. In the presence of 100 mM HU, both *pds1-128* and *mec1-1* cultures contain populations of cells with less than 1N DNA content: about 10% of the total cells after the first division (7 h after release from α factor) of both mutants; 21% for *pds1-128* after three generations (14 h after release); and 31% for *mec1-1* after three generations (14 h after release).

checkpoint control can be unequivocally demonstrated by measuring the relative timing of budding, DNA replication, spindle assembly and the onset of anaphase. Others have identified proteins required for checkpoint arrest when replication has been blocked [5]; we examined checkpoint control during ongoing DNA replication.

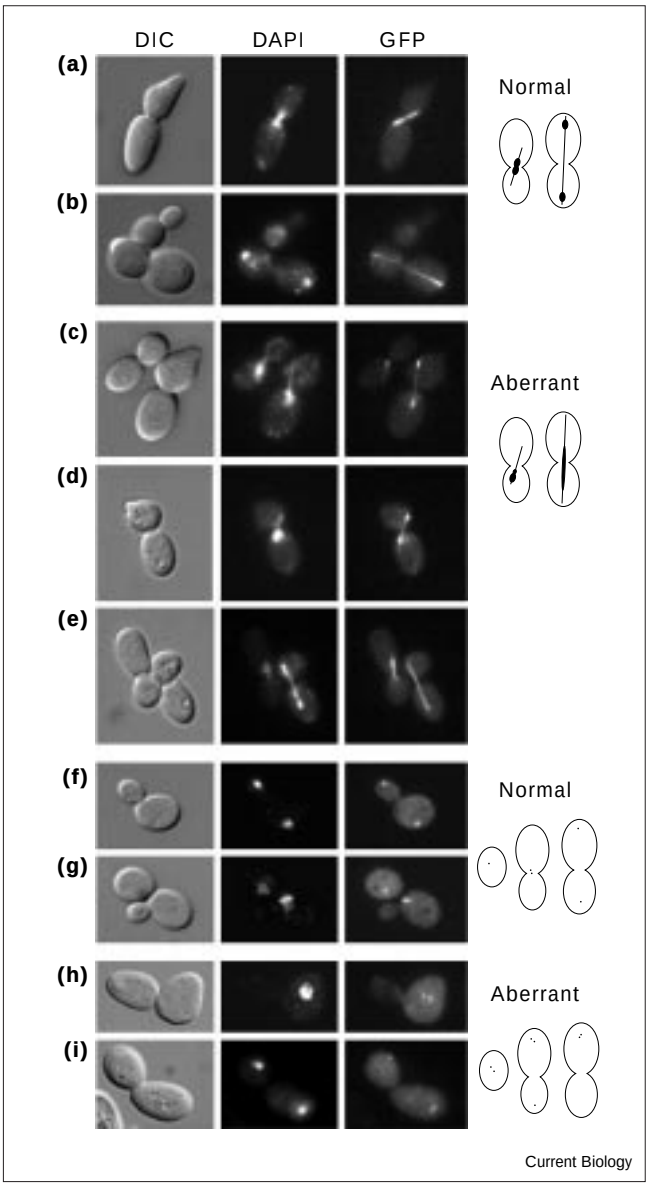
Wild-type and *pds1-128* cells were synchronized in G1 by adding α factor, then released into liquid YEPD medium containing 100 mM HU. To estimate the timing of the onset of anaphase, sister centromere separation was monitored (Figure 2a). Although both strains budded and progressed through S phase with similar timing, sister centromere separation was advanced in *pds1-128* cells. At least 36% of budded *pds1-128* cells had undergone sister centromere separation at a time when most cells were still in S phase, according to FACScan analysis (Figure 2b). Thus, *pds1-128* cells engage in premature sister centromere separation when the coupling of S phase and mitosis is challenged.

Other aspects of anaphase also occurred prematurely in the *pds1-128* mutants. For example, *pds1-128* cells elongated mitotic spindles about 2 hours before wild-type cells (Figure 3a), even though both strains replicated DNA with similar timing (data not shown). Considering the relatively short G2 interval in budding yeast, the 2 hour advancement of spindle elongation indicates that most *pds1-128* cells must have initiated anaphase before replication was complete. Indeed, FACScan profiles (data not shown, but see Figure 1) revealed that most cells had less than a 2N DNA content at a time when the bulk of the population had initiated spindle elongation. Wild-type and *pds1-128* cells progressed through S phase and began anaphase with indistinguishable timing in the absence of HU (see Supplementary material).

Uncoupling S phase from mitosis in *pds1-128* cells had several consequences. Following release from α factor in the presence of 100 mM HU, 50% of *pds1-128* cells engaged in an aberrant mitosis (Figure 4). After the first division, a population of cells with less than 1N DNA content was detectable by FACScan analysis (Figure 3d), and 30% of the newly divided cells exhibited gain or loss of the centromere region of chromosome IV (17% of cells had no centromere region IV signal, 13% had a signal >1; Figure 4), indicating that many nuclei failed to segregate evenly. After three generation times in HU, 50% of cells had an excess of centromere region IV signals and 21% had <1N DNA content. These abortive attempts at anaphase closely resemble those described for *scc1* mutants, in which sister chromatid cohesion fails to become established during S phase [8].

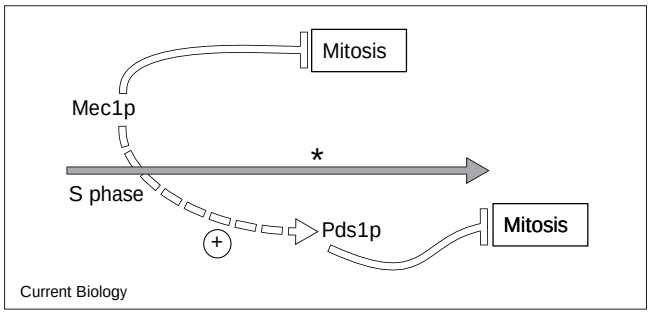
The sensitivity of *pds1* mutants to HU could partly reflect the DNA damage checkpoint defect of these cells because,

Figure 4



Aberrant mitosis part way through S phase in *pds1-128* cells. (a,b,f,g) Wild-type and (c–e,h,i) *pds1-128* cells grown in medium containing 100 mM HU. (a–e) Images are from differential interference contrast (DIC) microscopy, 4,6-diamidino-2-phenylindole (DAPI) staining to visualize the chromosomes, and fluorescence microscopy to visualize spindles (using GFP-labeled TUB1). (f–i) Images are from DIC microscopy, DAPI staining and fluorescence microscopy to visualize the centrosome of chromosome IV (using GFP-labeled tetR). (a) Normal early anaphase: spindle partly elongated, nucleus stretched at bud neck. (b) Normal late anaphase: spindle fully elongated, nucleus divided. (c,d) Aberrant early anaphases: spindles partly elongated, nuclei abnormally positioned and stretched. (e) Aberrant late anaphase: spindle fully elongated, nucleus not divided. (f) Normal late anaphase: nuclei divided, each has one GFP signal. (g) Normal early anaphase: nucleus at bud neck, slightly separated centromeres (adjacent GFP signals). (h) Aneuploid *pds1-128* cell: undivided nucleus away from bud neck; two GFP signals. (i) Aneuploid late anaphase *pds1-128* cells; one nucleus has two GFP signals.

Figure 5



Model for coupling replication with mitosis. Possible modes of S-phase checkpoint regulation: Mec1p and Pds1p are essential components of distinct and sequential checkpoint pathways that block mitosis, one active in early S phase, the other active part way through S phase, either operating in parallel (solid lines) or operating in series (additional broken arrow). Part way through S phase (gray arrow), there is a switch (*) in the mode of checkpoint control.

during S phase, HU inevitably causes replicative stress. The severe aneuploidy induced by HU treatment in *pds1* mutants suggests, however, that loss of coordination between replication and mitosis is responsible for the lethality. Indeed, *rad9Δ* cells, defective for all known aspects of DNA damage checkpoint control [9] but not sensitive to HU (Figure 1), were proficient at coupling S phase with mitosis, using the same experimental conditions as described above (Figure 3b). Therefore, the S-phase Pds1p-dependent checkpoint system is Rad9p-independent.

We have shown that Pds1p is required to couple ongoing replication with mitosis. An outstanding issue is why *pds1* mutants arrest efficiently when replication is completely blocked. One intriguing explanation could be that S phase and mitosis are coupled by distinct checkpoint pathways that act sequentially. To test this, we compared the checkpoint defect of *pds1-128* with that of *mec1*, the prototypic S-phase checkpoint mutant [5]. When the *mec1* checkpoint defect was analysed during partial inhibition of replication, we observed that *mec1* cells initiated anaphase as soon as spindle assembly occurred (Figure 3c). In contrast, although *pds1-128* cells began anaphase prematurely, there was a distinct period in early S phase when mitosis was restrained. This was also true of the *pds1* null mutant (see Supplementary material).

Rather than regulating mitotic kinase activity, the budding yeast DNA damage and spindle assembly checkpoints inhibit anaphase directly by stabilizing the inhibitor Pds1p [6]. This work demonstrates that Pds1p, as well as Mec1p, is essential to coordinate ongoing DNA replication with mitosis. Although both of these proteins are required, they must act at different times in S phase and in distinct pathways (Figure 5). Mec1p must function either independently of Pds1p throughout S phase, or independently of Pds1p in early S phase but upstream of Pds1p later in S phase. We propose that an event intrinsic to the progression of DNA replication elicits a switch in the mode of checkpoint regulation. As Pds1p is required for maintaining sister centromere cohesion, the Pds1p-dependent pathway may operate only once centromere cohesion has been established, an event that is likely to be completed by mid-S phase, when centromeric regions have been replicated [8,10]. Alternatively, Pds1p may be required following the initiation of late replication origins, because Mec1p was recently shown to be essential for an early S-phase checkpoint that inhibits late origin firing [11]. In response to DNA damage and spindle assembly checkpoint controls, Pds1p degradation is inhibited. By analogy, ongoing DNA replication may signal Pds1p stabilization in order to restrain anaphase until replication is completed.

Supplementary material

Additional methodological details, yeast strain genotypes and supplementary figures are published with this article on the internet.

Acknowledgements

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Supplementary material

The Pds1 anaphase inhibitor and Mec1 kinase define distinct checkpoints coupling S phase with mitosis in budding yeast

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Supplementary materials and methods

Strains are derivatives of BF264-*15Daub* [S1], except where stated. Cultures were grown at 26°C in YEPD medium (1% yeast extract, 2% bactopeptone, 50 mg/l adenine, 2% glucose), unless otherwise stated. Standard genetic procedures were used [S2]. The *pds1-128* strain was isolated in a mutational enhancer screen [S3]. Spindles were visualized by expressing a GFP–TUB1 construct [S4,S5]. The centromeric region of chromosome IV was visualized by utilizing the binding of tetR–GFP fusion proteins (expressed from the *CUP1* promoter) to tandemly integrated tetO sequences (at the centromere-linked *TRP1* locus); methodology similar to that described previously [S6]. FACScan analysis and microscopy were as described [S5].

Yeast strain genotypes

DCY1180, *MAT**a** bar1Δ*; DCY1167, *MATα pds1-128*; DCY1228, *MAT**a** bar1Δ pds1::KAN^R*; DCY1359, *MATα rad9::TRP1*; DCY1699, *MAT**a** bar1Δ mec1-1 ura3::HIS3:GFP:TUB1(URA3)*; DCY1698, *MAT**a** bar1Δ his3::HIS3:GFP-TUB1(HIS3)*; DCY1717, *MAT**a** bar1Δ rad9:TRP1 ura3::HIS3:GFP:TUB1(URA3)*; DCY1671, *MAT**a** bar1Δ ura3::HIS3:GFP:TUB1(URA3)*; DCY1672, *MAT**a** bar1Δ pds1-128 ura3::HIS3:GFP:TUB1(URA3)*; DCY1662, *MAT**a** bar1Δ CUP1:GFP:tetR(KAN^R) trp1::TRP1-(tetO)*; and DCY1663, *MAT**a** bar1Δ pds1-128 CUP1:GFP:tetR(KAN^R) trp1::TRP1-(tetO)*.

References

S1. Richardson HE, Wittenberg C, Cross FR, Reed SI: An essential G1 function for cyclin-like proteins in yeast. *Cell* 1989, 59:1127-1133.

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S5. Segal M, Clarke DJ, Reed SI: Clb5-associated kinase activity is required early in the spindle pathway for correct preanaphase nuclear positioning in ***Saccharomyces cerevisiae***. *J Cell Biol* 1998, 143:135-145.

S6. Straight AF, Belmont AS, Robinett CC, Murray AW: GFP tagging of budding yeast chromosomes reveals that protein-protein interactions can mediate sister chromatid cohesion. *Curr Biol* 1996, 6:1599-1608.

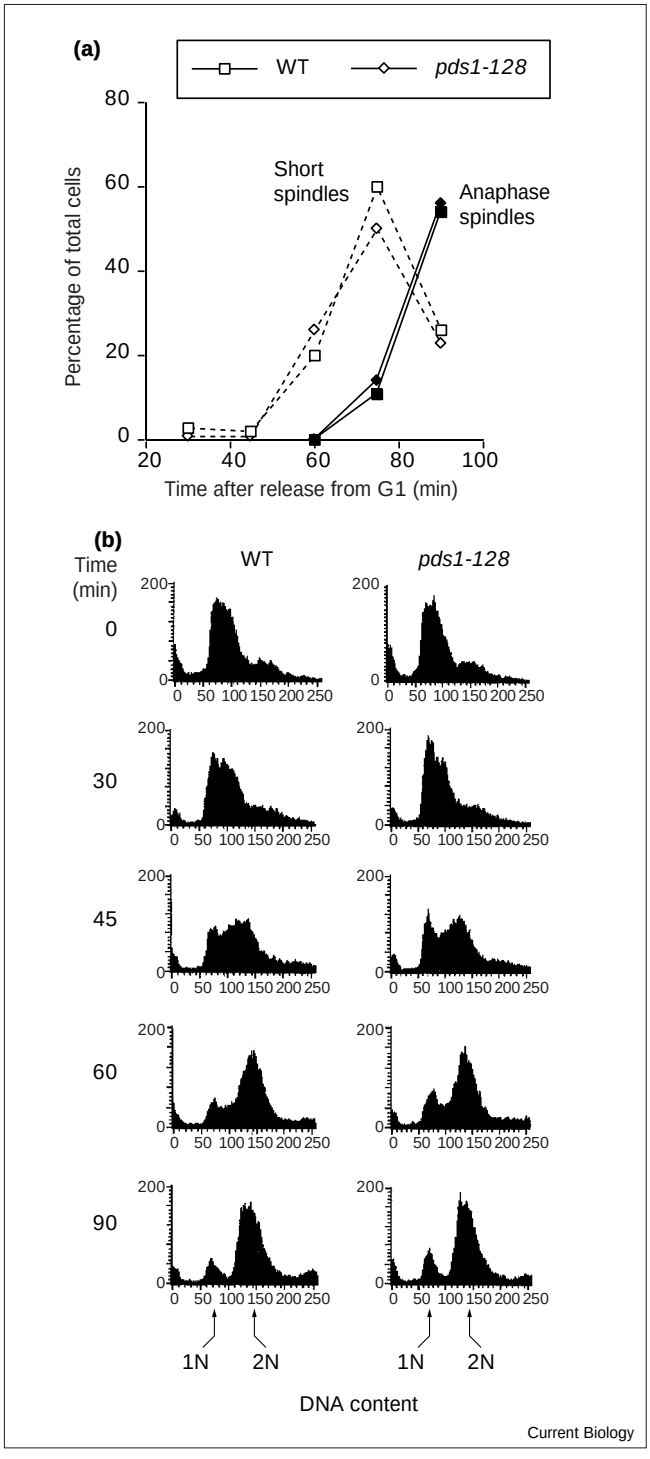
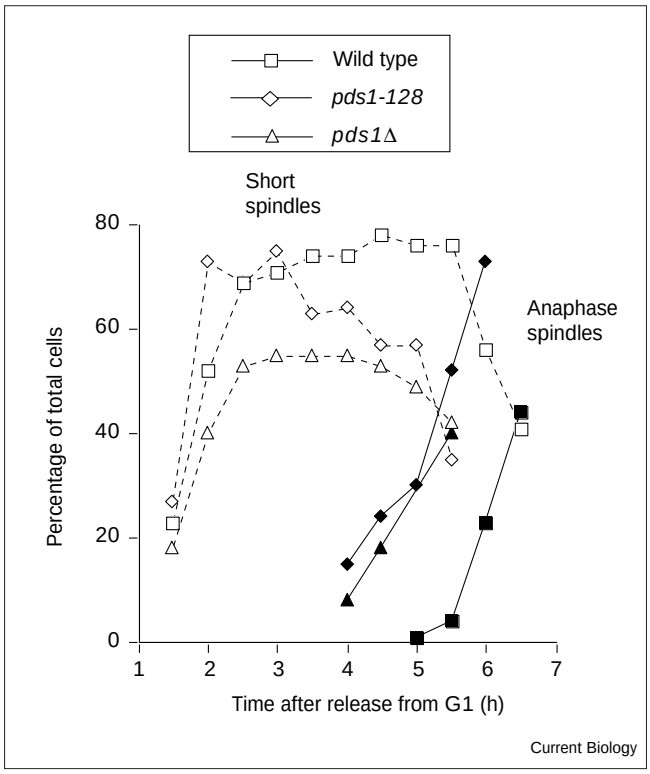


Figure S1

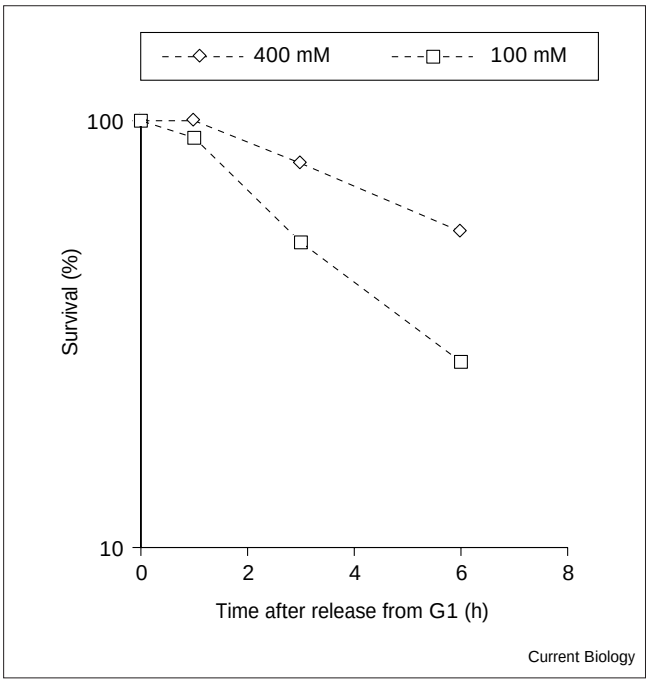
Kinetics of an unperturbed cell cycle in wild-type and *pds1-128* cells. Wild-type (WT) and *pds1-128* cells were arrested in G1 with 200 ng/ml α factor (at least 90% unbudded cells), then released into YEPD medium at 26°C. (a) Cell aliquots were taken at given time intervals for scoring short spindles (open symbols) and elongated spindles (black symbols), and for FACScan analysis of DNA content. (b) Histograms show the DNA content of cells in samples at selected time points from the time course (time following release from α factor is indicated for each sample on the left-hand side of each histogram). Each strain formed short spindles, replicated DNA, and elongated spindles with similar kinetics.

Figure S2



S-phase checkpoint control defects in *pds1-128* and *pds1Δ* mutants. Strains were arrested in G1 with α factor (at least 90% unbudded cells), then released into YEPD medium containing 100 mM HU at 26°C. Cell aliquots were taken at given time intervals to score short spindle formation (open symbols) and spindle elongation (black symbols), and for FACScan analysis of DNA content. Each strain replicated DNA with similar kinetics (data not shown).

Figure S3



Viability of *pds1-128* cells grown in liquid YEPD containing 100 mM or 400 mM HU. The *pds1-128* cells were arrested in G1 with 200 ng/ml α factor (at least 90% unbudded cells), then released into YEPD medium containing 100 mM or 400 mM HU at 26°C. Cell aliquots were taken at given time intervals for scoring colony formation on YEPD medium. FACScan analysis (data not shown) confirmed that replication was arrested with 400 mM HU. In 100 mM HU, cells replicated DNA with kinetics comparable to those presented in the main text of the article.